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ORIGINAL ARTICLE

# Endostatin combined with radiotherapy suppresses vasculogenic mimicry formation through inhibition of epithelial–mesenchymal transition in esophageal cancer

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**Abstract** The growth of solid tumors requires angiogenesis to provide oxygen and nutrients and to support cell proliferation. The switch from an avascular to a vascular phenotype is typically related to acceleration of tumor growth. Anti-angiogenic therapy is becoming a very promising way for malignant tumors. Meanwhile, malignant tumor cells themselves were able to develop the formation of cell-lined vessels that contribute to tumor neovascularization and supply the nutrients and oxygen, which is called vasculogenic mimicry (VM). However, the molecular mechanism of VM remains unclear. The purpose of this study was to investigate the efficacy of the novel recombinant human endostatin (rh-Endo) protein combined with radiotherapy on human esophageal squamous cell carcinoma (ESCC) cell lines Eca-109 and TE13. Our results showed that rh-Endo combined with radiotherapy significantly inhibited the proliferation, migration, invasion, and VM of human esophageal cancer cells in a dose-dependent manner; however, it has no direct effect on apoptosis of carcinoma cells, which indicated that rh-Endo combined with radiotherapy significantly changed the microenvironment of esophageal carcinoma, and played an important role in preventing distant metastasis. Our findings suggested that rh-Endo inhibited the metastasis of esophageal cancer and the activation of AKT pathway, and the down-regulation of epithelial–mesenchymal transition (EMT) may be associated with such effect of rh-Endo. These results also supported the bright prospect of rh-Endo combined with radiotherapy for clinical applications in the future.

**Keywords** Recombinant human endostatin · Vasculogenic mimicry · Radiotherapy · Esophageal carcinoma · Epithelial–mesenchymal transition

**Abbreviation**

| | |
|---|---|
| ESCC | Esophageal squamous cell carcinoma |
| RT | Radiotherapy |
| rh-Endo | Recombinant human endostatin |
| NSCLC | Non-small-cell lung cancer |
| EMT | Epithelial–mesenchymal transition |
| VM | Vaculogenic mimicry |
| DMSO | Dimethyl sulfoxide |
| DMEM | Dulbecco's modified eagle medium |
| FITC | Fluorescein isothiocyanate |
| CCk-8 | Cell counting kit-8 |
| PTEN | Phosphatase and tensin homolog deleted on chromosome ten |
| Akt | Protein kinase B PKB |
| GSK-3β | Glycogen synthase kinase 3β |
| GAPDH | Glyceraldehyde-3-phosphate dehydrogenase |

## Introduction

Esophageal cancer (EC) is the eighth most common cancer and the sixth most common cause of cancer-related death all over the world, and esophageal squamous cell carcinoma

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(ESCC) constitutes the major pathological type [1]. Though radiotherapy (RT) plays an important role in the local control of ESCC, the survival rate of patients with esophageal carcinoma still remains to be less than 20 %. The most probable reason is that the radiotherapy may not prevent the metastasis of the primary tumor, while the tumor's metastasis causes the death [2]. Angiogenesis, or the formation of new capillaries from preexisting blood vessels, is involved in the pathogenesis of various diseases such as cancer, atherosclerosis, and diabetic retinopathy [3]. Several recent researches showed that anti-angiogenesis therapy may become one promising treatment of these diseases [4, 5]. Recombinant human endostatin (rh-Endo, under the trade name of Endostar), a fragment of collagen XVIII, was mainly found in the basement membrane around blood vessels. It has previously been applied to treat atherosclerosis and was also pushed into anti-cancer clinical trials [3, 6]. The rh-Endo has been approved by the China Food and Drug Administration since 2005 for the treatment of non-small-cell lung cancer (NSCLC) [7, 8]. In our previous study, we found that rh-Endo inhibits the development of cancer in mice xenotransplant model of esophageal squamous carcinoma. Nevertheless, the molecular mechanism still remains ambiguous.

VM involves the tube formation of tumor cells consisting of a type of mesenchymal cell, similar to epithelial–mesenchymal transition (EMT) [9]. EMT is the process by which differentiated epithelial cells changes from an epithelial cobblestone phenotype to an elongated fibroblastic phenotype [10], which results in the decreased expression of epithelial markers such as E-cadherin and the increased expression of mesenchymal markers such as Snail and N-cadherin [11]. Currently, EMT has been implicated in the processes that tumor cells metastasize to distant area and acquisition of therapeutic resistance [12]. Recent studies suggest that EMT plays a crucial role in the progress of cancer radioresistance [13]. In our study, we tried to find out the direct effect of rh-Endo combined with radiotherapy on the migration, invasion, and the tube formation of esophageal cancer, exploring the possible molecular mechanism. The bright prospects in application of this experiment were also revealed here.

## Materials and methods

### Reagents

Human recombinant endostatin (endostatin, also known as "rh-Endo") was kindly provided by Simcere Pharmaceuticals (Nanjing, China).

Antibodies for phosphatase and tensin homolog deleted on chromosome ten (PTEN), Snail, GSK-3β, phosphorylated GSK-3β (p-GSK-3β), Akt, and phosphorylated Akt (p-Akt) were purchased from CST (Cell Signaling Technology, MA). E-cadherin antibodies were purchased from Abcam.

### Cell lines and cell culture

ESCC cell lines ECA109 and TE13 were obtained from the Shanghai Institute of Cell Biology (Shanghai, China) and were maintained in Dulbecco's modified eagle medium (DMEM) medium (Gibco, Life Technologies, Carlsbad, CA, USA) supplemented with 10 % fetal bovine serum (Hyclone, GE Healthcare, Little Chalfont, UK), 1 % penicillin/streptomycin (Invitrogen, Life Technologies). Cells were maintained in an incubator at 37 °C, in an atmosphere containing 5 % $CO_2$.

### Cell viability assay

A cell counting kit-8 (CCK-8) assay was used to measure the cell proliferation. Eca-109 and TE13 were seeded into 96-well plates at $5\times10^3$ cells/well for 24 h, then incubated with different concentrations of Rh-Endo (25, 50, 100, 200, 400, 600, and 800 μl). Then, a CCK8 cell proliferation and cytotoxicity assay kit (Obio Technology, Shanghai, China) was used after 24 h. The absorbance was measured at a wavelength of 490 nm.

### Clonogenic survival assay

Esophageal cancer cells ECA109 and TE13 cells were seeded onto 6-well dishes. After overnight culture, cells were treated with 6-MV X-rays at doses of 0, 2, 4, 6, or 8 Gy at 4.5 Gy/min. The cells were then cultured in a 5 % $CO_2$ incubator at 37 °C for 2 weeks. The colonies were fixed and stained with Giemsa dye to count the number of colonies (>50 cells/colony) under microscope.

### Flow cytometric analysis

ECA109 and TE13 cells were plated in 6-well plates at a specific density. The cells were treated with rh-Endo (100 and 400 μg/ml) and exposed to X-rays (6 Gy). After 24 h, the cells were fixed in 2 % paraformaldehyde and stained with an Annexin V-FITC Apoptosis Kit (Keygene Biotechnology, Nanjing, China). The apoptotic cells were detected by flow cytometry with Cell-Quest software (BD Biosciences, San Jose, CA, USA).

### Wound-healing migration assay

Tumor cells were seeded in 6-well plates and cultured until 60 % confluence; then, we treated each well with different concentrations of rh-Endo and different doses of IR. The confluent cell monolayer was scratched with a pipette tip to

generate a vertical line across the middle of the wells. The spread of wound closure was observed after 0 and 24 h intervals and was photographed by a light microscope.

### Transwell invasion assay

Invasion assays were performed in the transwell (Corning, MA, USA) chamber with 8-um pore size polycarbonate filters, which are coated with 2 mg/ml of basement membrane, and matrigel (Corning, MA, USA) was used. Five hundred microliters medium containing 20 % fetal bovine serum (FBS) were added to the bottom of the chambers as the chemotactic factor and then $1\times10^5$ tumor cells were seeded into the top chambers with 500 ul DMEM (without FBS). After that, we treated the top chambers with different concentrations of rh-Endo (100 and 400 μg/ml) or/and 6 Gy of IR, and then incubated for 24 h. The invasive cells were fixed with cold 4 % paraformaldehyde, stained with 0.1 % crystal violet for 15 min, and washed with PBS three times. The fixed cells were photographed under a light microscope (×100) from three random fields.

### 3D culture and tube formation assay

We used a 15-well μ-Slide (Ibidi, Germany) to conduct tube formation assay. Briefly, to promote cell adhesion, we filled the inner well with 10 μl liquid Matrigel and incubated them at 37 °C for 1 h. Forty microliters of cancer cell suspension ($2\times10^5$/ml) was seeded into each upper well and incubated at 37 °C and 5 % $CO_2$ as usual. The cultures were maintained in DMEM complemented with 10 % FBS and 0.1 % gentamicin sulfate. The cells then were retreated with altered concentrations of rh-Endo (0, 100, and 400 μg/ml) and different radiation doses (0 and 6 Gy). After 4 h, we photographed the tubular structure of cancer cells with an inverted microscope (OLYMPUS, Japan) at 100 magnification. We then counted the total tube length and the number of branch points in three random view fields each well using an Ibidi Quantitative Tube Formation Image Analysis-Wim tube system under a light microscope (×100).

### Western blot analysis

The total proteins were extracted from the tumor cells using SDS Lysis Buffer (Keygen, Nanjing, China). The contents of proteins were measured by BCA assay (Keygen, Nanjing, China). Equal amounts of protein from each lysate were separated by SDS-PAGE (10 % acrylamide) and blotted to PVDF Western blotting membranes (Millipore). The membranes were blocked, probed overnight with primary antibodies against PTEN, Akt, p-Akt, GSK-3β, p-GSK-3, E-cadherin, Snail and glyceraldehyde 3-phosphate dehydrogenase (GAPDH, as loading control of total proteins). The immunoblotted proteins were visualized with the Chemidoc XRS imaging system (Quantity One Quantitation software; BioRad Laboratories, Hercules, CA, USA) using ECL reagents, after that measured by the public domain NIH Image J Program.

### Data analysis

The mean ± standard deviation (SD) from triplicate assays was calculated, and the differences between treatment groups were determined using the ANOVA test. Statistical analysis was carried out by STATA 11.0 software (StataCorp, College Station, TX, USA) and Prism 5.0 software (GraphPad, La Jolla, CA, USA). $P<0.05$ was considered to be statistically significant.

## Results

### rh-Endo does not improve radiosensitivity of ESCC in vitro

To explore whether rh-Endo affected ESCC cells directly and whether the improved radioresponse was due to radiation-induced tumor cell death, a CCK8 assay and flow cytometry assay were used. Although in CCK-8 assay, higher concentrations of rh-Endo (200 μg/ml and higher) exerted small inhibitory effects on the growth of ESCC cell lines, overall, there were no clear time- and dose-dependent effects (Fig. 1a). Flow cytometry assay revealed that rh-Endo combined with IR did not induce apoptosis of the ESCC cell lines ECA109 and TE13 in comparison with IR (Fig. 1c, d). The same conclusion was obtained in our previous clonogenic survival assay (Fig. 1b). These data suggested that the effect of rh-Endo combined with radiotherapy was not due to direct tumor-cell killing, which indicated it did not enhance the radiosensitivity in vitro [14].

### Effect of irradiation on esophageal cancer cell migration, invasion and tube formation

The cells migration and invasion ability of irradiated esophageal cancer has been increased [15]. In our trait, we evaluated the migration and invasion ability of irradiated ECA109 and TE13 cells in vitro. Wound-healing assay showed that the irradiated cancer cells had significantly faster closure of wound area compared with non-irradiated cells (Fig. 2). Cell invasion of irradiated cancer cells was found to increase obviously, when compared with non-irradiated cells (Fig. 3). These results showed that the esophageal cancer cells migration and invasion ability were greatly increased after irradiation. Thus, irradiation may promote tumor metastasis, though radiation therapy itself had a strong antitumor effect in situ.

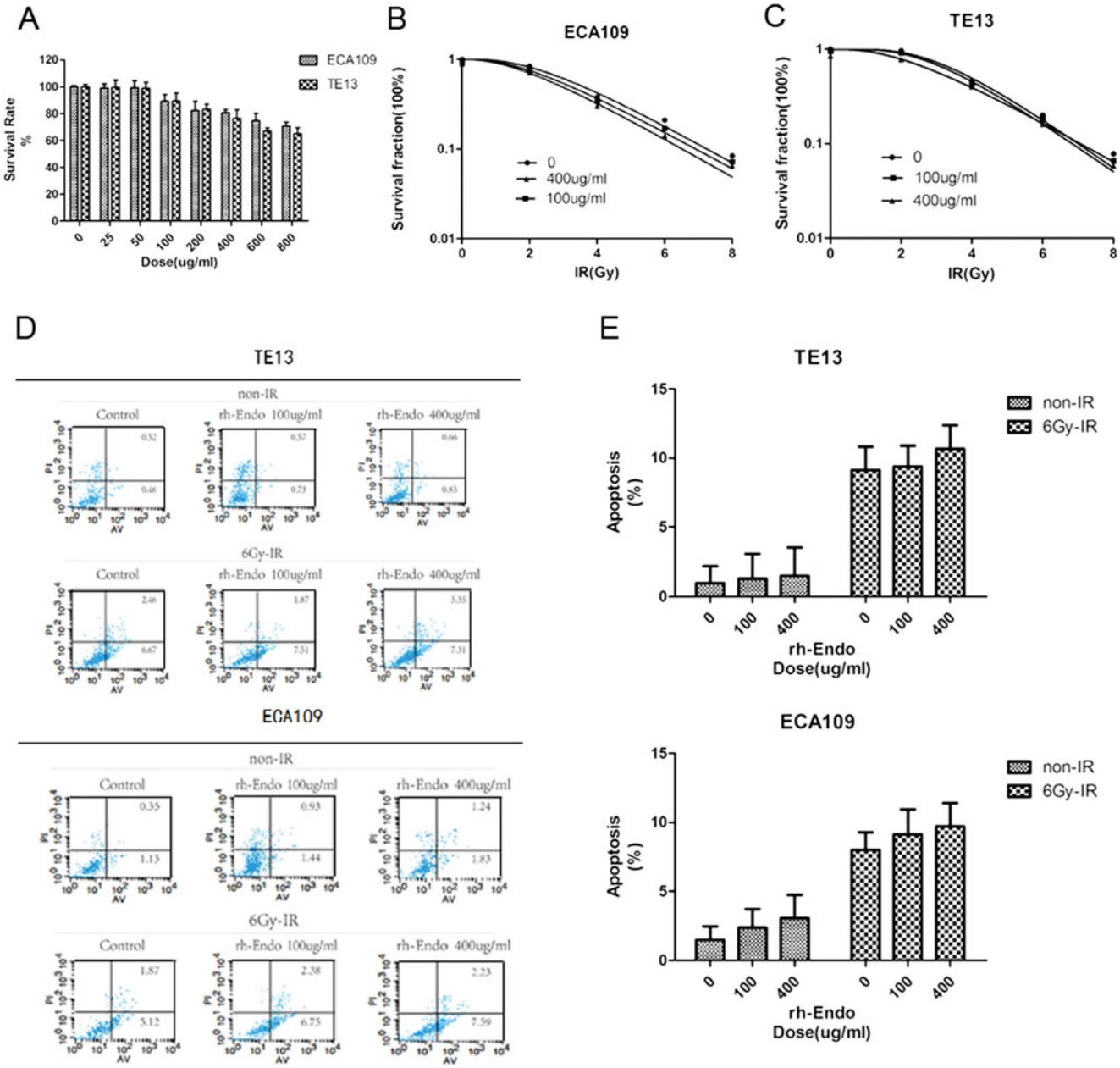


**Fig. 1** rh-Endo does not improve the radioresponsiveness of ESCC in vivo. **a** rh-Endo did not inhibit the proliferation of ESCC cells in a dose-dependent manner, and the experimental concentrations for ESCC cells ECA109 and TE13 were 100 μg/ml (as the lower concentration) and 400 μg/ml (as the higher concentration), respectively. **b**, **c** Cell survival curve was established by clonogenic survival assay. The cells were treated with 100 or 400 μg/ml rh-Endo and the doses of 2, 4, 6, and 8 Gy irradiation as illustrated and harvested after incubation for 10–14 days; the result showed no statistical significance. **d**, **e** rh-Endo (100 and 400 μg/ml) did not enhanced irradiation-induced apoptosis of ECA109 cells and TE19 cells; the result also showed no statistical significance

Tube formation (called as VM formation) of esophageal cancer cell is a key step in the change of tumor microenvironment. Therefore, we evaluated the effects of the tube formation of cancer cells. The result was the same as wound-healing migration assay and invasion assay. The ability of tube formation was strongly increased in irradiated groups compared with non-irradiated (Fig. 4).

## rh-Endo inhibits the migration and invasion of the non-irradiated and irradiated esophageal cancer cells

Wound-healing migration assays and transwell assays were used to further assess the effect of rh-Endo on the chemotactic motility of non-irradiated and irradiated cancer cells. rh-Endo significantly restricted the migration of cancer cells as

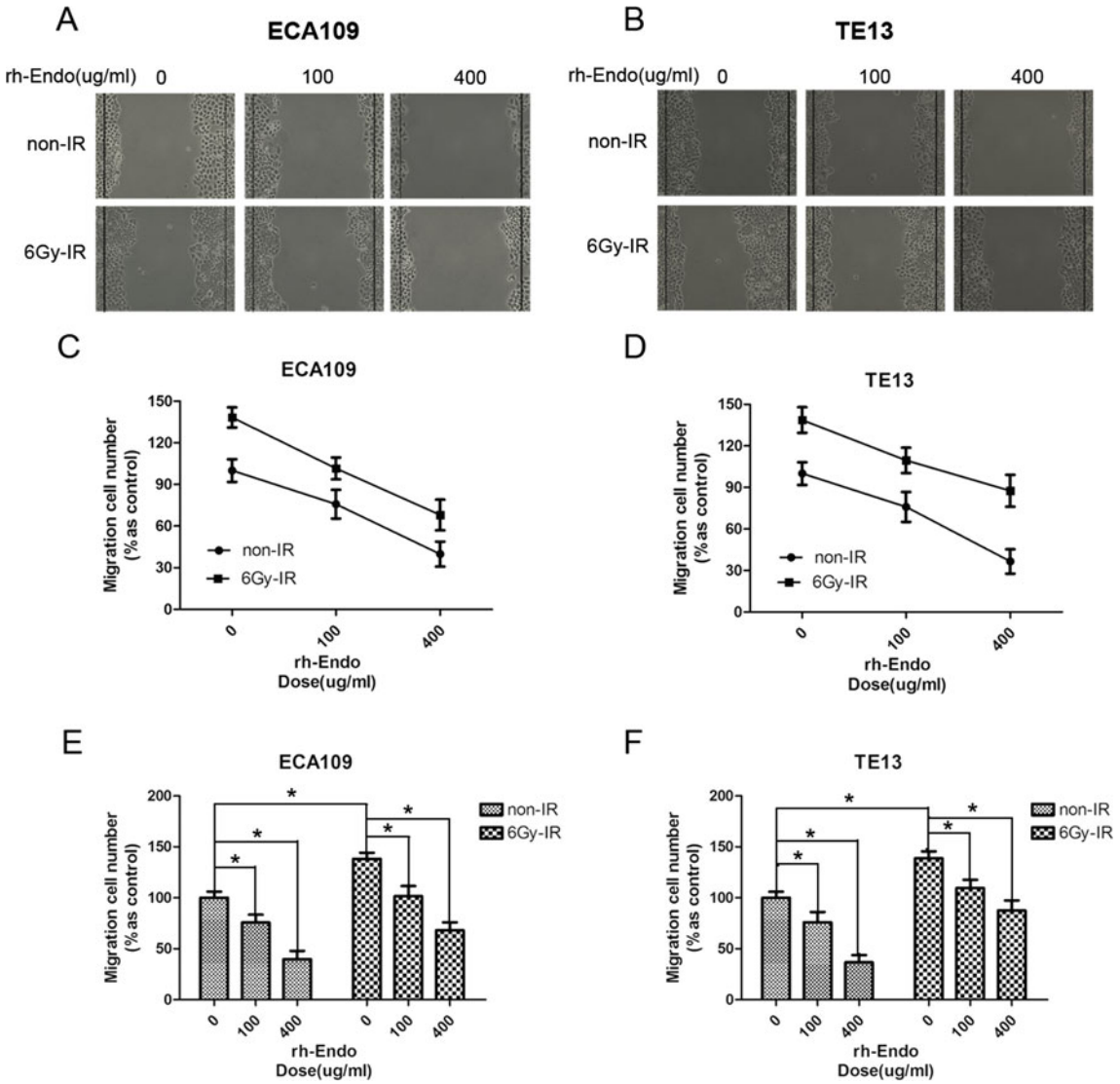


**Fig. 2** rh-Endo combined with irradiation inhibits ESCC migration in a dose-dependent manner. Cell migration was measured by wound-healing migration assay. ESCC were treated with different concentrations of rh-Endo and different doses of IR as illustrated, and the migrated cells were quantified by manual counting after 0 and 24 h using a light microscope. Data are expressed as the mean±S.E. ($n$=3).*$P$<0.05

presented in our wound-healing migration assays (Fig. 2). Meanwhile, in the invasive assays, only a few invasive cells were observed in the 400-μg/m rh-Endo-treated groups (Fig. 3). Therefore, we found that 100 and 400 μg/ml of rh-endo significantly inhibited the migration and invasion of cancer cells ECA109 and TE13 in both non-irradiated groups and 6-Gy-irradiated groups. The ratio was showed in the Fig. 4.

### rh-Endo restricts the tube formation of the non-irradiated and irradiated esophageal cancer cells

VM formation is involved in cell migration and invasion, and the mechanism of which is analogous to that of endothelial cells [9, 16]. Thus, we used a well-established in vitro model of 3D culture to investigate tube formation of esophageal cancer cells similar to VM formation. Cancer cells were incubated with 10 μl liquid Matrigel, and capillary tube structures formed in the each group (as described in the materials and methods) at 4 h. We found the total tube length and branching points of tube formation were considerably restricted by rh-Endo both in non-irradiated and irradiated groups (Fig. 4), which revealed that rh-Endo restricts VM formation of the esophageal cancer cells with a dose-dependent relationship.

### rh-Endo combined with irradiation inhibits migration, invasion, and VM formation of ESCC by inhibiting EMT thought inactivation of AKT/GSK-3β signaling pathway in vitro

Vasculogenic mimicry (VM) is a very complex process, related to the EMT, and mediated by many signaling routes, including the PTEN/AKT/GSK-3β signaling pathway [17, 18]. It has

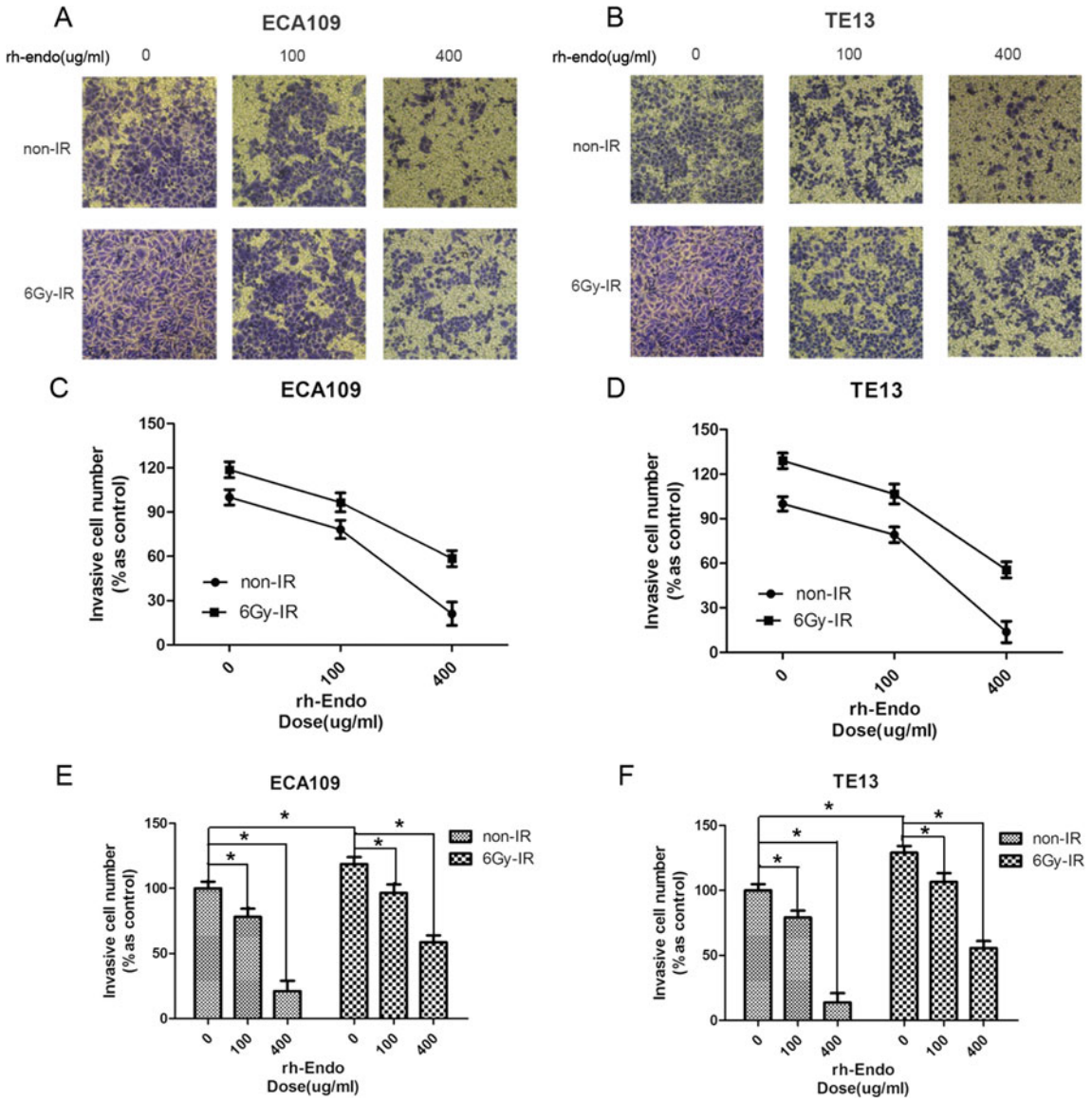


**Fig. 3** rh-Endo combined with irradiation inhibits ESCC invasion in transwell invasion assay. ESCC were seeded in the top chamber of transwell and treated with different concentrations of rh-Endo and different doses of IR. The bottom chambers were filled with 500 ml DMEM with 20 % FBS. After 24 h, the invasive ESCC were stained and quantified by manual counting. Data are expressed as the mean ±S.E. ($n$=3).*$P$<0.05

been reported that expression of PTEN is decreased after irradiation, and anti-angiogenic treatment inhibited cell proliferation and migration via regulating of the expression of local AKT [19, 20]. Thus, Western blot assays were performed to detect the protein expression of PTEN, total AKT, p-AKt, GSK-3β, and p-GSK-3β, and we found that the negatively regulate protein of AKT signaling pathway PTEN was decreased after irradiation, leading to the increase of Snail and the decrease of E-cadherin (two of EMT makers). Meanwhile, the expression of p-AKt and p-GSK-3β was increased remarkably. Most interesting was that all of these trends reversed when adding rh-Endo into each treatment group (Fig. 5). These results were obtained in both ECA109 and TE13, which suggested that PTEN deficiency activates Akt/GSK-3β/Snail/E-cadherin pathway upon irradiation, resulting in the increasing of migration, invasion, and VM formation of ESCC. While rh-Endo could reverse this process, leading rh-Endo combined with irradiation to becoming a promising approach to inhibition of the esophageal cancer cells metastasis.

## Discussion

Radiotherapy is a well-established treatment in esophageal cancer even at advanced stages. It provides survival benefits and the improvement in quality of life for esophageal cancer

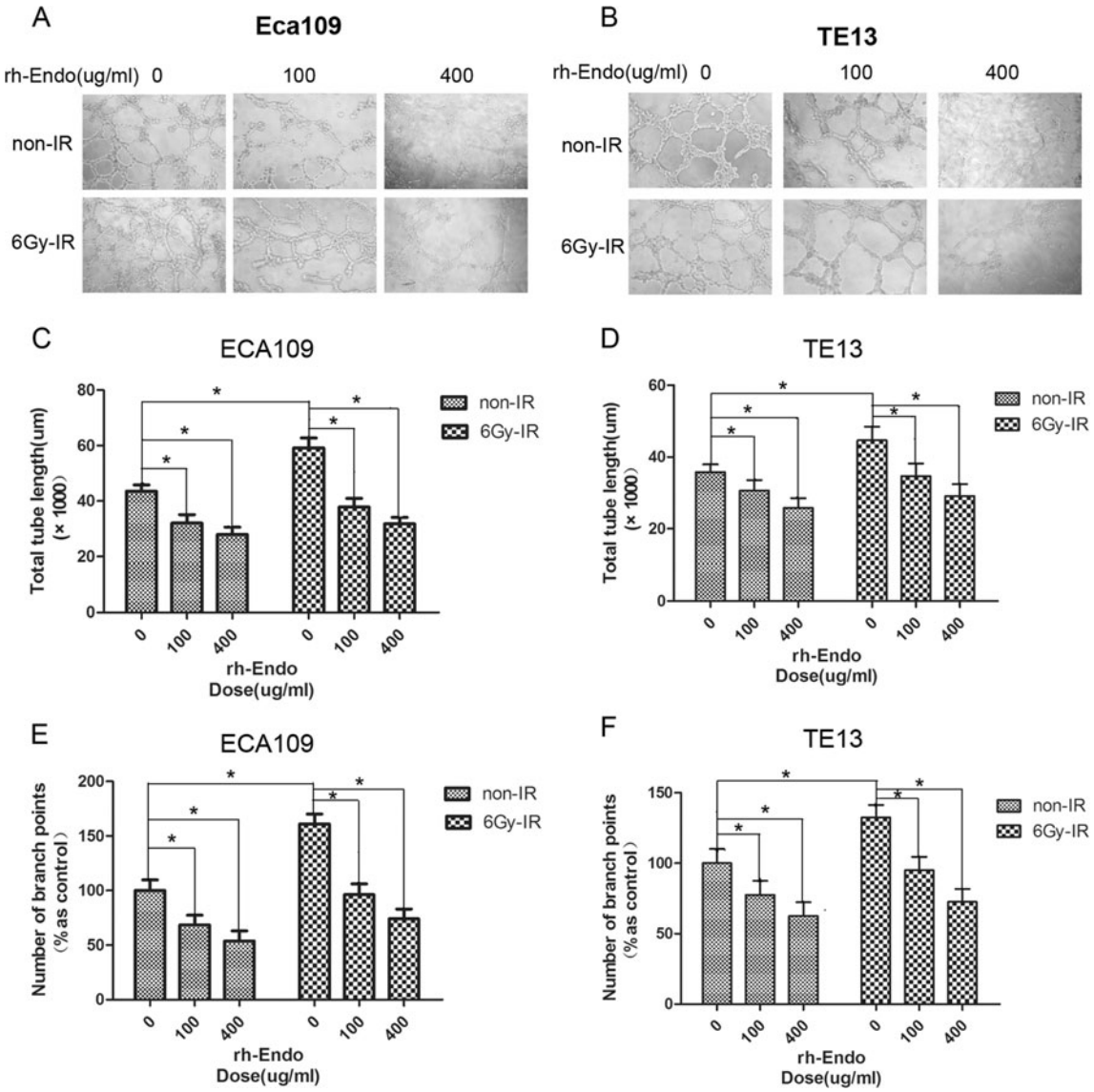


**Fig. 4** rh-Endo combined with irradiation inhibits VM formation of ESCC. HUVECs were seeded on a 15-well μ-Slide (Ibidi, Germany) at a density of $2\times10^5$ cells/well. After treated with different concentrations of rh-Endo and different doses of IR for 24 h, capillary tube structures were photographed (OLYMPUS, Japan), and the total tube length and the number of branch points in three random fields per well were counted. Representative capillary tube structures were shown. Data are expressed as the mean±S.E. ($n$=3).*$P$<0.05

patients, but the 5-year survival and the locoregional control of the disease are still not optimistic. The mechanism underlying the failure of radiotherapy may be caused by the metastasis of cancer cells [1, 2]. That rh-Endo is used as an anti-angiogenic agent in advanced NSCLC is suggested according to NCCN guidelines (Chinese version), which inhibited the cancer-related neovascularization of NSCLC, leading it to play an important role in the comprehensive treatment of NSCLC [21]. Recently, clinical trials and retrospective studies have demonstrated anti-tumor effect of rh-Endo in many other advanced tumors, such as metastatic melanoma, head and neck cancer, breast cancer, and gastric cancer [22]. However, it required more study to prove whether rh-Endo can be utilized in esophageal cancer. In our study, we demonstrated that rh-Endo could significantly inhibit EMT via decreasing PTEN expression and suppressing of the AKT/GSK-3β activity. Depending on the literature, irradiation promoted EMT in esophageal cancer cells through PTEN deficiency-mediated AKT activation, which may result in cancer cell metastasis and poor prognosis [15]. Therefore, rh-Endo combining with radiotherapy possibly provides more survival benefits than radiotherapy alone.

In the year of 2010 and 2011, two independent studies by Wang et al. and Maniotis et al. showed that tumors do not merely rely on the blood vessels of their hosts for nourishment and can form tube of cancer cells to make their own vasculature [23, 24]. These findings explain

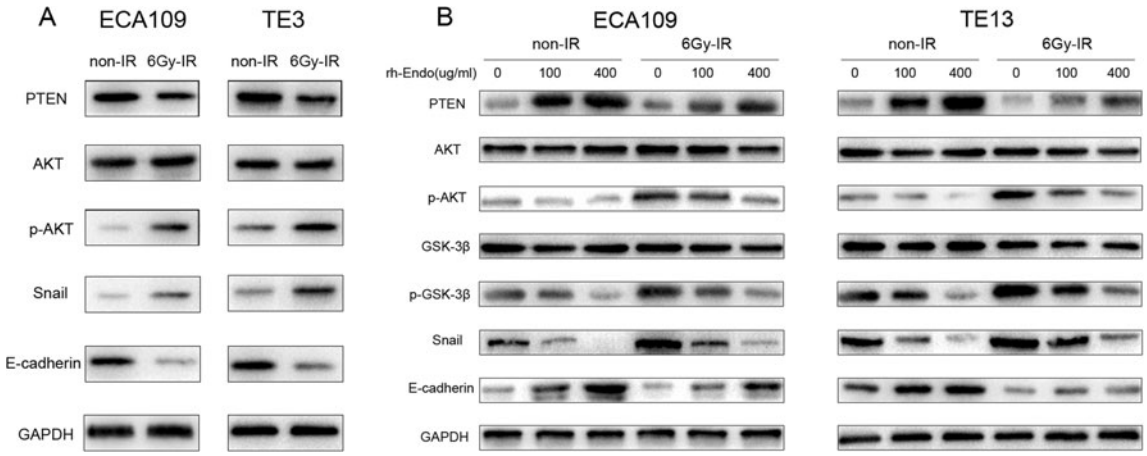


**Fig. 5** rh-Endo combined with irradiation inhibits EMT through inactivation of PTEN/AKT/GSK-3β signaling pathway in ESCC. **a** Western blot analysis showing that irradiation reduced the levels of PTEN and E-cadherin in ECA109 and TE13 cells and increased the levels of p-AKT and Snail, while the levels of AKT remained unchanged. **b** rh-Endo significantly increased the level of PTEN as well as E-cadherin and reduced the levels of p-AKT, p-GSK-3β, and Snail both in irradiated and non-irradiated groups. Meanwhile, the levels of PTEN and E-cadherin reduced and the levels of p-AKT, p-GSK-3β and Snail increased in irradiated groups compared with non-irradiated control groups ($P<0.05$)

why several treatments for cancer could not achieve the expected effect. It was not the first time that researchers suggested that cancer cells could form their own vessels. In 1999, Maniotis et al. reported a similar effect in melanoma cells, which they called vascular mimicry [24].In a recently study, Du et al. believed that hypoxia contributed to VM formation by inducing EMT [25]. Radiotherapy and anti-angiogenic therapy are all involved in the regulation of EMT [15, 26]. Our study partially revealed the relationship between irradiation, anti-angiogenic therapy, and EMT for the first time. Furthermore, we found that rh-Endo combined with irradiation cloud restrict VM formation of the non-irradiated and irradiated esophageal cancer cells via inhibiting the occurrence of EMT, which could provide a new sight for the comprehensive treatment of advanced esophageal cancer.

It is well known that a key step in EMT is down-regulation of E-cadherin. E-cadherin is regulated either by transcription factors, such as Snail-related zinc-finger transcriptional repressors (Snail and Slug), SIP-1/ZEB-2, Twist, or ubiquitylation-induced endocytosis. In our study, we found irradiation increased the expression of Snail and decreased the expression of E-cadherin at the protein level. Ectopic over expression of Snail also leads to the acquisition of increased resistance to apoptosis and cancer stem cell-like properties in various epithelial cells [27]. The activation of Snail may be one of mechanisms involved in the development of radioresistance in ESCC after radiotherapy. However, to our surprise, the expression of Snail was significantly decreased when combined with rh-Endo (the result was compared with irradiation alone group), which proved rh-Endo would promote the effect of radiotherapy though down-regulating the expression of Snail.

AKT pathway plays a central role in the three major radioresistance mechanisms, including intrinsic radioresistance, tumor-cell proliferation, and hypoxia. PTEN, as an inhibitor of AKT, is reported to associate with radiosensitivity [28]. Kim et al. found PTEN to be imperative for attenuation of invasion and EMT [29]. Covered together, the down-regulation of PTEN is linked with dephosphorylation of AKT and GSK-3β, and PTEN-dependent PI3K/Akt/GSK-3β signaling is necessary to up-regulate Snail and down-regulate E-cadherin for radiation-induced EMT in ESCC. In this study, we also found that rh-Endo could influence all the above process, including inhibiting the expression of PTEN, activated AKT/GSK3β pathway, decreased Snail, and increased E-cadherin. As a consequence, the EMT to develop in ESCC was inhibited by rh-Endo. This effect of rh-Endo may be a potential cellular and molecular mechanism for anti-angiogenic therapy and radiotherapy. It will have profound significance for future tumor-related comprehensive treatment.

The invasion and VM formation of esophageal cancer cells increased after irradiation. Meanwhile, rh-Endo could reverse this process. Regardless of the fact that we found the PTEN/AKT/GSK-3β signaling pathway involved, how the irradiated cancer cells sent messages to the cells surrounded remains to be further studied. Membrane-derived extracellular vesicles (EVs) (i.e., exosomes) are small vesicles with sizes ranging from 30 to 120 nm. Exosomes vary in cell type and mechanism of biogenesis [30]. Knowledge of secretome components that influence EMT including secreted proteins/peptides and exosomes has emerged. Exosomes mediate intercellular communication and direct integral facets of carcinogenesis that include EMT, invasion,

migration, angiogenesis, and metastasis [31, 32]. In another research of ours, exosomes were extracted from irradiated esophageal cancer cells supernatant, then added these exosomes to non-irradiated cancer cells. To our great surprise, the ability of VM formation and invasion in ESCC were promoted compared with a control group, which indicated exosomes the irradiated cancer cells secreted were involved in the EMT caused by radiotherapy. How exosomes regulate the EMT after irradiation needs more experimental results to reveal.

In conclusion, this study provided in vitro evidence that rh-Endo combined with radiotherapy inhibited migration, invasion, and VM formation of ESCC, indicating that rh-Endo probably suppresses metastasis of esophageal cancer, though it did not enhance the radiosensitivity of ESCC directly. This phenomenon is associated with the up-regulation of expression of PTEN and E-cadherin, down-regulation of expression of p-AKT, p-GSK3β, and Snail. We will continually find the value of rh-Endo combined with radiotherapy on clinical application.

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**Compliance with Ethical Standards**

**Conflicts of interest** The authors declare that they have no conflicts of interest.

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